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Inoculation with Biofilm of *Bacillus subtilis* Is a Safe and Sustainable Alternative to Promote Tomato (*Solanum lycopersicum*) Growth

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Abstract: Over-fertilization and agrochemicals adversely affect soil quality and agricultural ecosystem sustainability. Tomato (*Solanum lycopersicum*) is ranked as an important crop due to its high profitability and nutritional value. In Argentina, tomato is mainly produced in horticultural belts at peri-urban areas, whose soils frequently are contaminated by heavy metals and/or agrochemicals. To explore safer alternatives, we investigated the effects of seed inoculation with a common plant-growth-promoting rhizobacteria (PGPR), i.e., *Bacillus subtilis* subsp. *spizizenii*, on development at various growth stages of two tomato varieties, "Platense" and "Río Grande", and on production and fruit quality at harvest time of the "Río Grande" variety. The experimental design consisted of three treatments per variety: a control versus traditional planktonic or biofilm inoculation, with three replicates per treatment. Germination at 10 days and seedling agronomical parameters showed that the response to seed inoculation was superior in the "Río Grande" variety. At harvest time, and irrespective of the inoculant, several agronomical parameters of the "Río Grande" variety were significantly enhanced with respect to the control. The biofilm significantly increased tomato production, as quantified by fruit number and weight, compared to the planktonic inoculum. This case study demonstrates that the incorporation of bio-inoculants is relevant in sustainable agriculture to promote crop growth and quality.

Keywords: seed inoculation; bio-fertilizer; planktonic inoculum; biofilm; tomato; sustainable environment

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1. Introduction

Climate-change-induced abiotic (heavy metals, drought, salinity, organic matter decline, etc.) and biotic (crop pathogens, weeds, etc.,) stresses have been demonstrated to give rise to adverse effects on agricultural productivity [1,2]. In addition, the use of agrochemicals has led to threats to food security and soil quality [2,3]. On the other hand, there is a need to increase crop production worldwide to meet future food demands for a growing population [4–6]. Therefore, to cope with global climatic change coupled with a growing world population and increasing consumer demands for agrochemical-free food, agricultural practices should link two vitally important functions: productivity and sustainability. Subsequently, it is essential that environmentally friendly technologies are

developed to meet food demands and to improve the physicochemical and nutritional soil conditions, avoiding the risks associated with excessive agrochemical application [7].

The use of microorganisms as bio-inoculants is becoming increasingly important because they are capable of promoting plant growth, increasing soil quality and suppressing different soil-borne diseases [8–10]. In particular, PGPR bacteria (plant-growth-promoting rhizobacteria) have emerged as bio-fertilizers, phytostimulants and biocontrollers [11,12]. These can work as biological fertilizers to promote nutrient acquisition by several mechanisms, for example, biological nitrogen fixation, phosphate solubilization, potassium adsorption, iron sequestration, etc., [13–16]; also, they can protect plants from pathogenic microorganisms by releasing antibiotics, lytic enzymes and other metabolites [17–20] or by inducing resistance under stresses [2]. Therefore, the incorporation of beneficial PGPR, inoculated as bio-fertilizers, is becoming increasingly relevant in sustainable agriculture and is now viewed as a tool that may contribute to food safety and environmental health [7,21]. Among PGPRs, an important genus is *Bacillus* [14], which has been shown to be able to grow in various environments and to produce beneficial substances for plants. Several species of *Bacillus*, in particular, *B. subtilis* subsp. *spizizenii*, have been found to act as bio-fertilizers, phytostimulants and biological control agents [20,22,23].

Biofilms consist of three-dimensional structures that protect cells from adverse environmental conditions. The biofilm structure embodies a matrix of exopolysaccharides, with smaller amounts of proteins, DNA and various lysis products. Within the biofilm, different cell types coexist, including vegetative cells and bacterial spores, the latter being considered as resistant forms. Due to its spatial structure, biofilms facilitate the dispersal of spores in the environment [24–26]. A major trait of *Bacillus* lies in its potential to develop a biofilm at the air–liquid interface. Moreover, *B. subtilis* subsp. *spizizenii* can either develop a biofilm attached to a substrate or a surface or live as a free planktonic form depending on the cultivation environment [27,28]. Subsequently, this PGPR can be inoculated both as a biofilm or as a liquid in the conventional planktonic mode.

Tomato (*Solanum lycopersicum* L.) is ranked as the highest-yielding vegetable in the world, with 189×10^6 tons of fresh fruits produced in 2021 [29], covering a harvested area of over 5×10^6 ha. Fresh tomato fruits are generally eaten and prepared like a vegetable, while several processed products such as sauces, purees and juices are also ready to consume. In addition to its high profitability, this crop also is important because of its nutritional value and benefits for human health [30]. This is owing to the fact that tomatoes are rich in the major dietary source of the antioxidant lycopene, a carotenoid pigment; also, they are a great source of vitamin C, potassium, folate and vitamin K [31,32].

Peri-urban areas surrounding large metropolitan settlements may behave as green belts; intensive vegetable cultivation in this environment has the competitive advantage of being close to the consumer market [33]. Peri-urban soils, however, are not always the optimal suitable crop substrate because often they have a poor structure and heterogeneous surface horizons resulting from mixing the top and deep layers during tillage; frequently, these soils also present high concentrations of organic pollutants and/or heavy metals. This is the case in the Buenos Aires urban fringe as well [34]. Within the Argentinean horticultural sector, tomato is one of the most important vegetable crops, with a per capita consumption of around 16 kg/person/year. Its production reached 7.67×10^5 tons in 2020, and the estimated harvest surface was at about 1.18×10^4 ha [35]. When cultivated in peri-urban areas, this crop is subjected to several soil-borne diseases. Open-field cultivation of horticultural crops, including tomato and most significant grain crops in Argentina, makes use of commercial bio-inoculant formulations, which are commonly employed as an agro-ecological practice.

The inoculation or imbibition of tomato seeds and/or seedlings with *Bacillus* sp., and other similar PGPR have been previously studied [36–39]. However, while the use of bio-inoculants as a sustainable choice to avoid or shorten agrochemical demand has been extensively documented, fewer works have evaluated its effects on crop productivity. Moreover, until now, the majority of experiments were conducted using the traditional

planktonic form. To reduce these gaps, seed inoculation treatments with *Bacillus* sp., both in the planktonic form and as a biofilm, were investigated. We hypothesized that tomatoes treated with biofilm will have a higher yield and quality compared to those subjected to a planktonic treatment. Therefore, the current study aimed to (i) characterize inoculants from *B. subtilis* subsp. *spizizenii* prepared both as a biofilm and as a liquid in the conventional planktonic form and (ii) to evaluate and compare the effects of these two inoculant forms on growth, production and quality of two different varieties of tomato.

2. Materials and Methods

2.1. Biofilm and Planktonic State Characterization and Inoculant Preparation

The bacterium *B. subtilis* subsp. *spizizenii* used in the current investigation was deposited in the AGRAL collection located at the Faculty of Agronomy, Buenos Aires University (FAUBA). The bacterium culture corresponded to an American Type Culture Collection (ATCC) strain, accession number 6633. This subspecies was selected based on its beneficial effects on soil microbial activity and vegetable growth [20]. The bacterium from the stock culture was activated in nutritive agar media at 30 °C for 24 h.

2.1.1. Vegetative Cells and Spores in Biofilm and Planktonic State

The culture medium of the *B. subtilis* subsp. *spizizenii* was a liquid minimal salt medium (MSM) with 1% glycerol and 35 mM L-glutamic acid. The MSM contained 1 g/L K_2HPO_4; 0.3 g/L KH_2PO_4; 0.5 g/L NH_4Cl; 0.1 g/L NH_4NO_3; 0.1 g/L Na_2SO_4; 0.01 g/L $MgSO_4$ $7H_2O$; 1 mg/L $MnSO_4$ $4H_2O$; 1 mg/L $FeSO_4 7H_2O$; 0.5 g/L $CaCl_2$ and 0.01 g/L EDTA in deionized water at pH = 7 [40].

Bacteria populations in a planktonic state were produced from a culture grown at 30 °C with agitation at 150 rpm for 96 h in a rotatory agitation incubator. To produce biofilm, cultivation was carried out under static conditions at 30 °C for 96 h. Figure S1 in the Supplementary Materials shows an example of biofilm at the air–liquid interface.

We determined the number of spores and vegetative cells in both the planktonic state and in the manually disaggregated and homogenized biofilm. Determination of vegetative cells was performed with the serial dilution method and colony count on a nutritive agar plate. Spore determinations in the planktonic state and in the disaggregated biofilm involved heating at 80 °C for 1 h before performing the serial dilution method [41].

2.1.2. Biofilm Stability

B. subtilis subsp. *spizizenii* was cultivated in MSM with 55 mM L-glutamic acid and either 1% glycerol or 1% glucose as a carbon source. Then, 150 mL of the inoculated culture medium was incubated in a 250 mL Erlenmeyer flask and maintained under static conditions at 30 °C for 96 h. Biofilms developed in the inoculated culture medium at the air/liquid interface were used for stability estimations.

To assess the stability over time, biofilms developed in MSM were kept at room temperature (23 ± 2 °C) and were removed and dried at 40 °C to constant weight every 5 days. To evaluate stability at different temperatures, the biofilms obtained in MSM were maintained either at 23 ± 2 °C, room temperature, or at 4 °C; again, the biofilms were removed every 5 days and processed in the same way.

2.1.3. Inoculant Preparation

Biofilm and planktonic inoculants were produced by the procedure outlined in Section 2.1.1. Thus, again, *B. subtilis* subsp. *spizizenii* was cultivated in liquid MSM with 1% glycerol and 35 mM L-glutamic acid. To obtain populations of bacteria in a planktonic state, cultures were grown at 30 °C for 96 h under continuous stirring at 150 rpm in a rotatory agitation incubator. To prepare biofilm-inoculant-based treatments, cultures were grown at 30 °C for 96 h under static conditions.

2.2. Experimental Site and Design

First, a seed germination assay in the laboratory was conducted, and then greenhouse trials were carried out. Then, two independent greenhouse assays were performed to acquire the agronomic parameters at the end of the vegetative growth stage (50 days after seed planting) and at harvest time. Laboratory and greenhouse facilities were located at the Faculty of Agronomy, University of Buenos Aires (FAUBA) (34°45′ S latitude, 60°31′ W longitude).

Two varieties of tomato (*S. lycopersicum*) were studied, namely, Platense and Río Grande. The former is an indeterminate and the second a determinate variety type; indeterminate varieties show a trend of achieving the flowering stage and producing fruits later than determinate types. Commonly, both varieties are commercially produced in Argentina.

A completely randomized design with three treatments was used. The treatments consisted of (a) inoculation with the planktonic form, (b) the application of biofilm and (c) a control. In the control treatment, non-inoculated seeds were used, and subsequently, the experiment was conducted in a similar way to the inoculated treatments. The number of replicates per treatment depended on each particular assay and will be given hereafter.

All laboratory and greenhouse experiments were conducted under natural light. They took place over a 4-month time span, from October to February, during the austral spring and summer.

2.2.1. Seed Germination Assays

Seeds of Platense and Río Grande tomato varieties were disinfected by washing, first with 70% alcohol and then three times with sterile distilled water. A layer of sterilized cotton covered with sterile filter paper with a pore size equivalent to Whatman Grade 3 was placed in sterile Petri dishes and moistened with 5 mL of sterile distilled water.

Ten seeds were placed in each Petri dish and maintained under dark conditions at 22 °C. In the planktonic culture treatment, each seed was inoculated with 0.1 mL of destilled water. In the biofilm treatment, the seeds and biofilm were mixed, taking advantage of their high adherence. In the control treatment, the seeds were infused with 0.1 mL of distilled water. Each treatment had five replicates.

Observations were made 5 and 10 days after inoculation without uncovering the boxes, and a visible radicle length of at least 2 mm was the criterion for germination occurrence [42]. Then, the germination percentage (G%) was determined according to Araya et al. [43].

$$G\% = \left(\frac{germinated\ seeds}{total\ seeds}\right) \cdot 100 \quad (1)$$

Moreover, 15 days after seed inoculation, the radicle and hypocotyl length in each seedling were also measured.

2.2.2. Greenhouse Assays

Two successive greenhouse assays were carried out for two tomato phenological stages. The first assays involved both the Platense and Río Grande tomato types and the length was limited to the vegetative growth stage, i.e., 50 days. With the information obtained from this experiment, a second assay was established, lasting for the complete growth cycle until harvest time, which solely involved the Río Grande variety. In each assay, the three treatments mentioned in Section 2.2. were investigated. The temperature in the greenhouse was, on average, 30 ± 5 °C.

Seeds of the studied tomato types, i.e., Platense and Río Grande in the first assay and Río Grande in the second assay, were soaked for 30 min using either planktonic or biofilm inoculants. Subsequently, they were sown in seedling trays with cells of 5 cm in diameter and 10 cm in depth, which contained a mixture of commercial substrate and compost in a 3:1 ratio. The commercial substrate had a 50% humidity; its pH was 5.8; on a dry basis, it consisted of 55% organic matter and 45% ash, and its C/N ratio was 30% [44]. Twenty-five seeds per treatment were placed in the respective cells.

In the first assay, 15 days after seed inoculation, 25 seedlings per treatment of the two studied varieties were transplanted into 3 L pots, placing one seedling per pot and filling with the 3:1 mixture of commercial substrate and compost, as before, at an average temperature of 24 °C. Plants were grown for 50 days, which corresponded to the end of the vegetative growth stage, just before flowering.

Next, the Río Grande variety was chosen to perform the second assay. Again, a set of seeds were inoculated and grown in pots as above described using 25 seeds per treatment. Then, after 15 days, the seedlings were transplanted into 7 L pots, and the trial continued for 4 months until fruit development, ripening and harvest.

2.3. Agronomic and Production Variables

At the end of the vegetative stage, i.e., after growing for 50 days, the plants were harvested and dried in an oven at 70 °C until constant weight. Then, the plants were separated in terms of shoot and root biomass, and each part was weighed.

At harvest time, in addition to dry shoot and root biomass, the fruits were measured and weighed in the different treatments. Also, fruit quality was evaluated in the fruit juice from total soluble solids (Brix) determinations, which were carried out using a hand-held refractometer.

In each of the three treatments studied, leaf area (LA) at harvest time was evaluated from 25 plants of *S. lycopersicum*, Río Grande variety. For each plant, 10 entire and fresh leaves were sampled from successive branches of the central area of the canopy; the selected leaves were of a medium size, and no more than a leaf per branch was taken. Each leaf was put over a white background and fully expanded by covering it with a 3 mm wide glass; then, it was photographed. The area of each leaf was estimated using the IMAGE J software 1.8.0. for scientific image analysis [45].

2.4. Chlorophyll Estimations

To quantify chlorophyll in the Río Grande variety at harvest time, two discs of 1 cm in diameter were cut from the central area of selected leaves. Three plants per treatment were sorted out, and determinations were made in three representative leaves sampled from the central part of the canopy of each selected plant. The discs were placed in an Eppendorf flask, and 1.5 mL of dimethylformamide was added; thereafter, they were left for 48 h in a refrigerator in the dark.

Absorbance was measured at 664 nm, 647 nm and 652 nm for quantification of chlorophyll a, chlorophyll b and total chlorophyll [46], respectively, in an Agilent 8453 UV–visible spectrophotometer. Chlorophyll contents were reported on a wet basis as mg chlorophyll/g fresh tissue.

The following equations were used for the quantitative estimation of the different chlorophyll types:

$$\text{Chlorophyll a} = 12.7 \times \text{Abs } 664 - 2.79 \times \text{Abs } 647 \quad (2)$$

$$\text{Chlorophyll b} = 20.7 \times \text{Abs } 647 - 4.62 \times \text{Abs } 664 \quad (3)$$

$$\text{Total chlorophyll} = 17.9.\ \text{Abs } 647 + 8.08.\ \text{Abs } 652 \quad (4)$$

where Abs 664, Abs 647 and Abs 652 are the absorbance at 664, 647 and 652 nm, respectively.

2.5. Bacterial Counts in Root Extracts of Solanum lycopersicum var. Río Grande

The method described by Dobereiner et al. [47] was used to assess the number of bacteria inside the roots. Determinations were made in plants sorted out at harvest time. Root samples of 10 g were taken and superficially disinfected by immersion in a 70° alcohol solution for 10 min and by shaking at 200 rpm; then, the solution was transferred to sterile Erlenmeyer flasks with a 3% sodium hypochlorite solution for 10 min and with shaking at 200 rpm; finally, the samples were washed 3 times with sterile water to remove traces of sodium hypochlorite for 10 min and once with sterile saline. The tissues were homogenized

in sterile saline, and CFUs were determined by serial dilution of the supernatant on a nutrient agar medium. Roots from non-inoculated plants were used as controls.

2.6. Statistical Analysis

Data were subjected to a one-way analysis of variance (ANOVA). Means were compared using Tukey's test to identify significant pair-wise differences at $p < 0.05$.

3. Results

3.1. Content of Vegetative Cells and Spores in the Planktonic State and in Biofilm

Most suitable inoculants developed to be used as a bio-fertilizer are expected to incorporate a high number of both vegetative cells and spores. Therefore, first total counts of vegetative cells and spores were made. The estimated populations varied depending on the culture medium, as shown in Figure 1. The planktonic inoculant exhibited about 10^{12} cells, while the biofilm counts resulted in about 10^{9} cells. The number of spores counted in the two inoculant forms were almost identical, in amounts of about 10^{6}. Spores are resistant structures that can remain dormant for long periods under non-favorable conditions; later, spores may again produce vegetative cells if the conditions are appropriate for germination. Therefore, bacterial spore richness is important in that it ensures microorganism viability over time as long as the spores remain a source of vegetative cells.

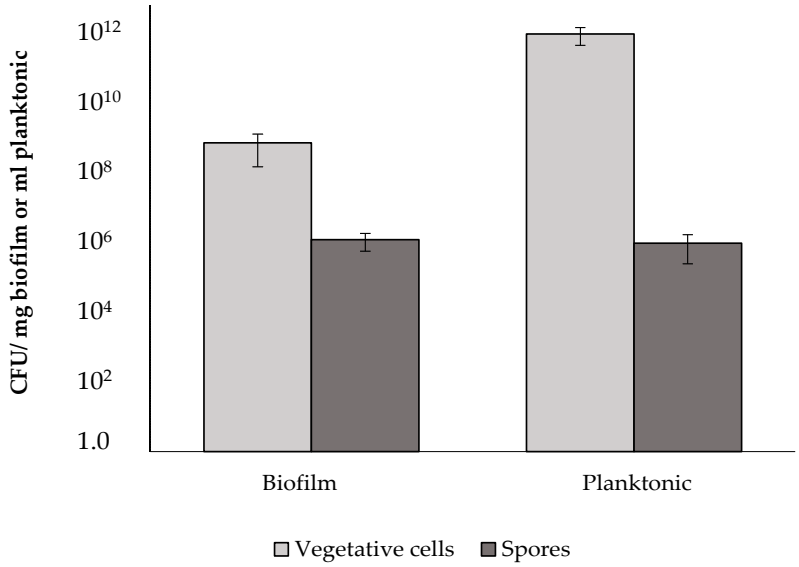

Figure 1. Populations of vegetative cells and spores of *B. subtilis* subsp. *spizizenii* used either as a biofilm (in static conditions) or as planktonic inoculum (at 150 rpm). Culture medium was MSM plus 55 mM L-glutamic acid and 1% glycerol at 30 °C during 96 h. Error bars represent standard deviations ($n = 3$).

The proportion of spores to vegetative cells was higher in the biofilm than in the planktonic inoculant. However, the opposite was true in absolute terms, so that for the biofilm, the count of vegetative cells was three orders higher than that of spores, while for the planktonic state, the count of vegetative cells was six orders higher than that of spores.

3.2. Biofilm Stability

3.2.1. Effect of Carbon Source

Figure 2 shows the biofilm stability as a function of the carbon source. The air–liquid interface biofilms, obtained at 25 °C, were much more stable using 1% glycerol than using 1% glucose.

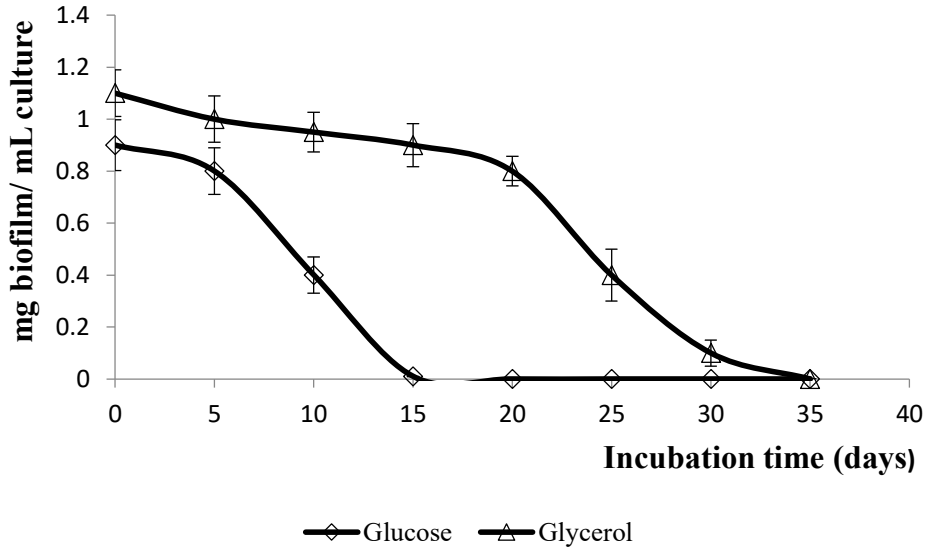

Figure 2. Stability curves of biofilms grown in MSM and 55 mM L-glutamic acid plus either 1% glycerol or 1% glucose. Error bars represent standard deviations (n = 3).

The biofilm gained from glycerol was stable for 20 days, and it then began to break up until it completely discomposed by day 35. In contrast, the biofilm obtained from glucose only was stable for 4 days, and it was completely disintegrated in by day 15.

3.2.2. Effect of Temperature

The effect of temperature was studied on the biofilm obtained using glycerol as a carbon source. As shown in Figure 3, the biofilm that developed at the air–liquid interface at 25 °C started to slowly disintegrate by day 12, and this trend was intensified by day 25; total degradation was observed by day 40. In contrast, the biofilm kept at 4 °C started to disintegrate by day 30, and only 36% of the biofilm had degraded by day 40, as counted from the start of the trial.

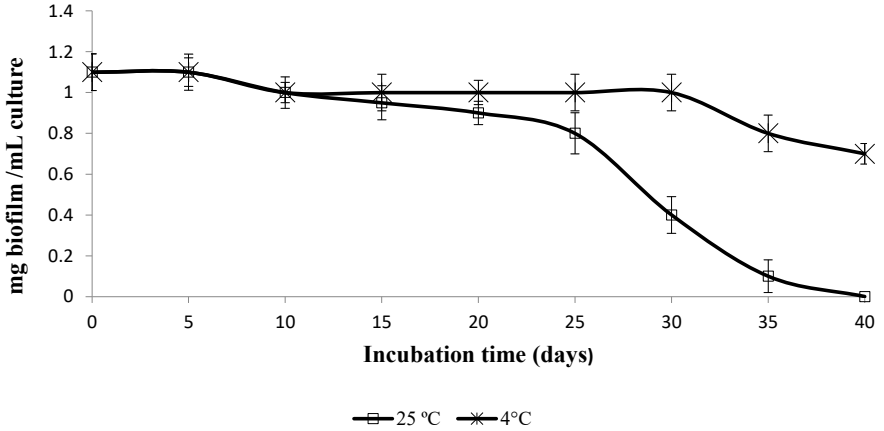

Figure 3. Effect of temperature on biofilm stability grown in MSM and 55 mM L-glutamic acid plus 1% glycerol. Error bars represent standard deviations (n = 3).

The higher biofilm stability at 4 °C suggests that bacteria cultured at low temperatures could be a good strategy to increase the lifespan of the biofilm inoculum.

3.3. Effects of Inoculation Form on Germination and Growth of Two Varieties of S. lycopersicum

3.3.1. Effects on Germination Percent of Platense and Río Grande Varieties

The germination percentages of the two varieties of *S. lycopersicum* inoculated either with biofilm or with the planktonic form obtained from *B. subtilis* subsp. *spizizenii* are shown

in Table 1. The varieties evaluated were Platense and Río Grande, while the germination tests were performed by days 5 and 10 after inoculation. Regardless of time and treatment, the Platense variety showed a lower germination rate.

Table 1. Effect of seed inoculation treatment, i.e., planktonic form or biofilm of *B. subtilis* subsp. *spizizenii*, on germination percentages of *S. lycopersicum* at days 5 and 10. Mean and standard deviations are given (n = 5). Different lowercase letters indicate differences between treatments at the 0.05 probability level.

	Germination (%) 5 Days			**Germination (%) 10 Days**		
Tomato varieties	**Control**	**Planktonic**	**Biofilm**	**Control**	**Planktonic**	**Biofilm**
Platense	74.2 ± 1.3^{b}	82.4 ± 0.8^{a}	27.0 ± 0.9^{c}	80.3 ± 2.1^{b}	87.0 ± 0.9^{a}	90.1 ± 0.9^{a}
Río Grande	88.0 ± 1.1^{a}	90.0 ± 1.5^{a}	30.0 ± 1.1^{b}	90.4 ± 1.4^{b}	95.2 ± 1.3^{a}	96.0 ± 0.8^{a}

After 5 days, the germination percentages per treatment were ranked as follows: planktonic inoculation > control > biofilm inoculation, so that the biofilm application resulted in a slowing down of the germination capacity of the two studied varieties. Moreover, by day 5, the percent germination of both the Platense and Río Grande varieties inoculated with the planktonic form were 8% and 5% higher than their specific controls, respectively. This meant that the differences were significant ($p < 0.05$) in the former variety and non-significant ($p < 0.05$) in the latter variety (Table 1).

Irrespective of the inoculation treatment, the maximum percent germination for the two varieties was recorded by day 10. At this time, the germination percentages of the Platense and Río Grande varieties were ranked as follows: biofilm > planktonic > control. There were significant differences between the control and the two seed inoculation treatments ($p < 0.05$). However, no significant differences were found between the planktonic and biofilm treatments (Table 1).

3.3.2. Effects on Seedlings of Platense and Río Grande Varieties after Germination

Inoculation of *S. lycopersicum* seeds with *B. subtilis* subsp. *spizizenii* showed a clear effect on seedlings growth of the Platense and Río Grande varieties compared to the control; the magnitude of this effect varied, depending on the application mode, i.e., planktonic or biofilm. In the Platense variety, both the planktonic and biofilm inoculants yielded increases in radicle length with respect to the control treatment of 14% and 32%, respectively; the radicle length differences between the inoculated and control treatments were not significant ($p < 0.05$) using the planktonic form, while they were significant ($p < 0.05$) using the biofilm. The radicle length increments in this variety with respect to the control were 3.3% and 10% using the planktonic and biofilm inoculants, respectively, and did not show any significant differences between the treatments. Therefore, the biofilm application to the Platense variety was demonstrated to be more efficient than the planktonic inoculation, so that the former treatment resulted in a 15% larger radicle and a 7% larger hypocotyl compared with the latter treatment (Figure 4a,b).

Greater seed inoculation effects were observed on the seedlings of the Río Grande variety; in this case, the inoculation using the planktonic and biofilm inoculum resulted in significant ($p < 0.05$) increments in the radicle length of 25% and 57%, respectively, with respect to the control (Figure 4a). The hypocotyl extension of the Río Grande variety was also significantly higher ($p < 0.05$) when the inoculated treatments and the control treatment were compared, so that increments of 20% and 41% for the planktonic and for biofilm treatments, respectively, were recorded (Figure 4b). Again, the biofilm inoculation was most effective in the Río Grande variety, with increments of 25% in the radicle and 18% in the hypocotyl lengths, respectively, relative to the planktonic form (Figure 4a,b).

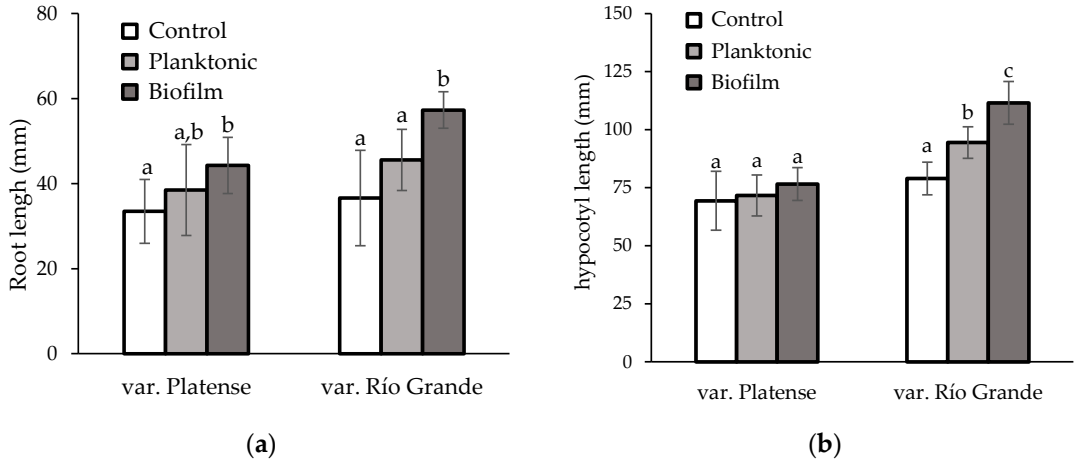

Figure 4. Effect of seed inoculation treatment on root (**a**) and hypocotyl (**b**) length of seedlings from Platense and Río Grande varieties from *S. lycopersicum*. Differences between lowercase letters indicate differences at the 0.05 probability level.

3.3.3. Effects on Biomass of Platense and Río Grande Varieties at the End of Vegetative Growth Stage

After 50 days of plant growth, the end of the vegetative growth stage was achieved in the two varieties studied; at this stage, neither the indeterminate Platense variety nor the determinate Río Grande variety still showed signs of flowering. The two inoculation methods produced significant increments in root development compared to the non-inoculated control in the Platense treatment; the planktonic cell inoculation resulted in a 62% increase, while biofilm application resulted in an 80% increase in root biomass on a dry weight basis. Although the greatest root development was observed after the biofilm application, the differences between both inoculation methods were not statistically significant (Figure 5a).

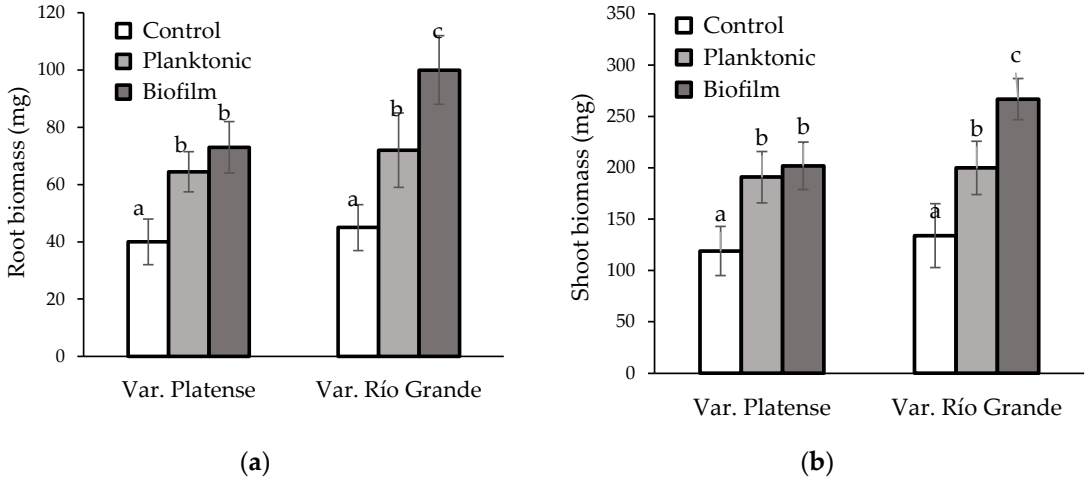

Figure 5. Effect of seed inoculation treatment on root (**a**) and shoot (**b**) biomass at day 50 of Platense and Río Grande varieties from *S. lycopersicum*. Differences between lowercase letters indicate differences at the 0.05 probability level.

Also, in the Río Grande variety, the two inoculation methods showed significant differences ($p < 0.05$) when compared to the control treatment; the shoot dry biomass became 60% and 120% greater when using the planktonic film and the biofilm, respectively. In contrast to the Platense variety, the Río Grande variety showed significant differences ($p < 0.05$) between the inoculation methods, so that the biofilm application resulted in a 30% higher shoot biomass than the planktonic inoculation (Figure 5b).

In addition, the seed inoculation with *B. subtilis* subsp. *spizizenii* produced a positive effect on the shoot biomass of *S. lycopersicum*. In the Platense variety, the shoot biomass increments were as high as 58% and 66% when implementing the planktonic inoculum and the biofilm, respectively (Figure 5b). However, no significant differences between inoculation methods were found.

The shoot biomass of the Río Grande variety showed increases with respect to the control that were as high as 48% when applying the planktonic inoculum and as high as 103% when applying the biofilm. Again, the shoot biomass was significantly different ($p < 0.05$) between the inoculation methods, so that it was 38% higher using the biofilm than using the planktonic inoculum.

In summary, the growth of *S. lycopersicum* seedlings inoculated with *B. subtilis* subsp. *spizizenii* assessed by day 50 depended on the plant variety and the inoculation method. The Río Grande variety performed better than the Platense variety regarding root and shoot biomass, while the biofilm was superior to the planktonic inoculum.

3.4. Effects on Production and Quality of Río Grande Variety at Harvest Time

Based on the results obtained with seedlings of different ages, the Río Grande variety was selected to continue the experiments for assessing the effect of seed inoculation with the planktonic form and the biofilm on several parameters of plant production and quality at harvest time.

3.4.1. Effects on Plant Biomass and Plant Height

Significant ($p < 0.05$) root biomass increments of 75% and 125% were achieved when comparing the planktonic and biofilm inoculation, respectively, with the control. Both inoculations using either a planktonic form or a biofilm showed significant ($p < 0.05$) increments of 40% and 80% in shoot biomass compared to the control, respectively. Also, significant ($p < 0.05$) differences between the inoculation treatments were observed, showing that the biofilm was the most effective method, as both the root and the shoot biomasses of this treatment were 29% higher than those of the planktonic treatment (Figure 6).

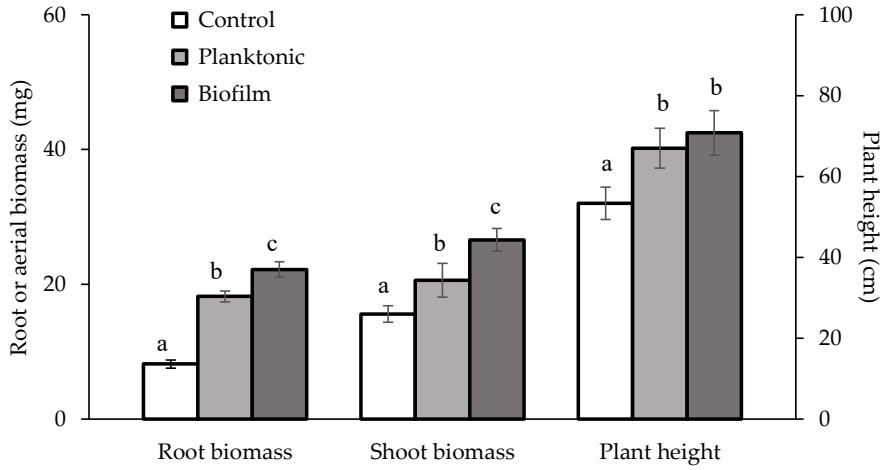

Figure 6. Effect of seed inoculation treatment on root and shoot biomass and on plant height at harvest time of Río Grande variety from *S. lycopersicum*. Differences between lowercase letters indicate differences at the 0.05 probability level.

With regard to plant height, the inoculated treatments (planktonic and biofilm) also showed significant ($p < 0.05$) differences when compared to the control treatment; however, no significant differences were found between the inoculation methods.

Figures S2–S4, presented in the Supplementary Materials, illustrate the differences at harvest time between selected plants from the different treatments. Figures S2 and S3 show the planktonic inoculum alongside the control plants and the biofilm alongside the control

plants, respectively, while Figure S4 compares the planktonic inoculum and biofilm plants. The size differences between the inoculated and control treatments are very apparent.

3.4.2. Quantification of Chlorophyll and Leaf Area

Chlorophyll, as measured in various forms, is the main photosynthetic pigment in higher plants, and its leaf content mainly depends on leaf N concentration, which governs the maximum photosynthetic activity. Plants of the Río Grande variety, whose seeds had been inoculated with the bacterium both in its planktonic form and as a biofilm, did not show significant differences at harvest time in terms of their chlorophyll a, chlorophyll b and total chlorophyll levels with respect to plants whose seeds had not been inoculated (Figure 7).

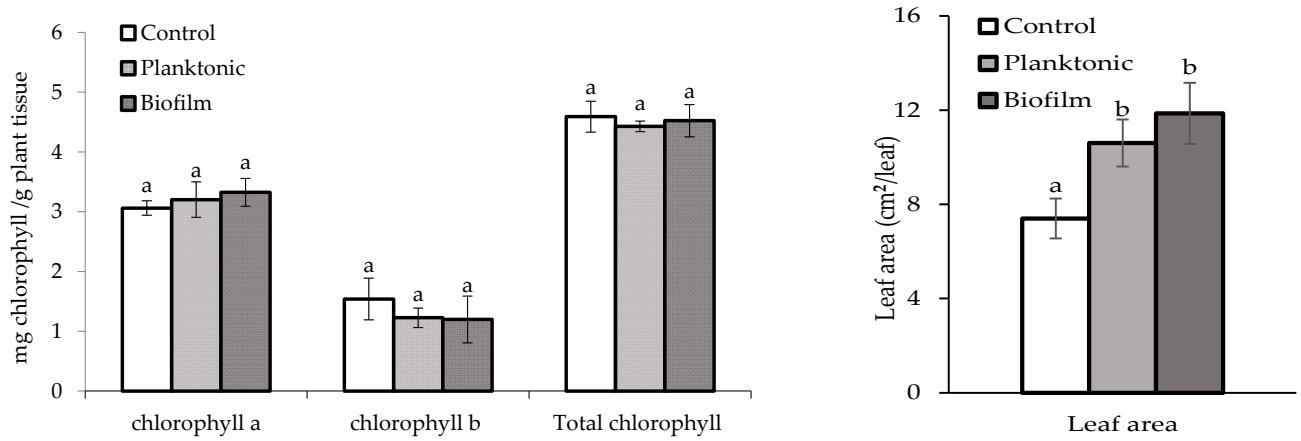

Figure 7. Effect of seed inoculation treatment on chlorophyll a, chlorophyll b and total chlorophyll contents (**a**) and leaf area (**b**) at harvest time of Río Grande variety from *S. lycopersicum*. Differences between lowercase letters indicate differences at the 0.05 probability level.

Leaf area was estimated from measurements in selected leaves from different tomato branches sampled with the same scheme in the three treatments and was measured in cm^2/leaf. Indeed, the mean LA determined in this work cannot be compared with the commonly used leaf area index, LAI, a dimensionless magnitude. We assumed that the LA may also be related to photosynthetic primary production. The mean LA values were 7.4, 10.6 and 11.8 cm^2/leaf. Therefore, the tomato plants growing from inoculated seeds showed significantly higher ($p < 0.05$) mean LA values than those growing from non-inoculated seeds. Even if the biofilm treatment showed the highest LA value, no significant differences with respect to the planktonic form of the treatment were found (Figure 7b).

Also, it was remarkable that the tomato plants inoculated with the biofilm had a greater number of leaves and branches compared to those treated with the planktonic inoculum, which was qualitatively estimated from Figure S4; this is important, as plant architecture can affect plant biomass partitioning and fruit yield.

3.4.3. Fruit Productivity and Quality

A clear-cut parameter for appraising tomato productivity is the produced number of fruits. The number of fruits harvested showed significant ($p < 0.05$) differences between the inoculated plants and control treatments; differences with respect to the control were as high as 33% for the planktonic treatment and 180% for the biofilm treatment. Moreover, the application of the biofilm prevailed over the planktonic inoculum, as the former treatment resulted in a 35% larger fruit number compared to the latter (Figure 8a).

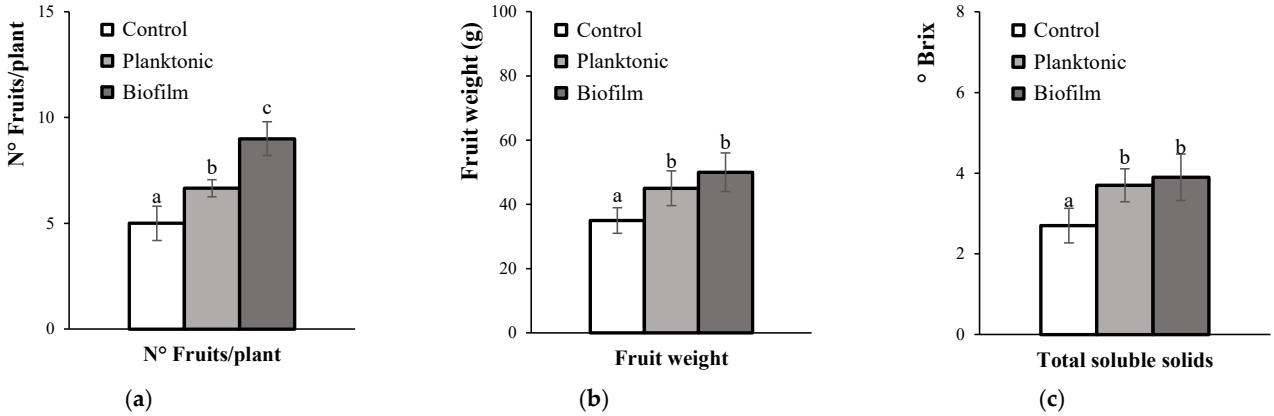

Figure 8. Effect of seed inoculation treatment on number of fruits (**a**), mean fruit weight (**b**) and total soluble solids (**c**) at harvest time of Río Grande variety from *S. lycopersicum*. Differences between lowercase letters indicate differences at the 0.05 probability level.

With regard to fruit weight, the planktonic and biofilm treatments showed 29% and 43% increases, respectively, compared to control, but the differences between the two seed inoculation methods were not significant.

In addition, the carbohydrate content of the fruit is one of the most important tomato quality attributes, both for fresh and processed consumption. In addition, the levels of soluble sugars contribute strongly to the soluble solids content and flavor of tomato fruit; therefore, the determination of the concentration of total soluble solids (Brix) is considered a quality parameter.

The results showed significant ($p < 0.05$) differences between the inoculated plants and the control that were as high as 37% for the planktonic treatment and 44% for the biofilm treatment, with no significant differences observed between the treatments (Figure 8c). Moreover, the total soluble solids of the fruits developed with the application of the biofilm almost reached the optimum reference value (4 Brix) for fresh tomato consumption.

3.5. Bacterian Endophytism

The population of *B. subtilis* subsp. *spizizenii* in the roots of *S. lycopersicum* var. Río Grande was counted at harvest time, i.e., after 4 months of seed inoculation. A total of 2×10^4 CFU/g of fresh root weight was unveiled in the roots of the plants growing from the seeds inoculated with the biofilm, while no bacteria were recovered from the roots of the control treatment. This may indicate that the biofilm inoculation allowed the bacteria to colonize roots and to implement its positive effects on plant growth, pointing at *B. subtilis* stimulation by environmental signals after seed germination. However, conclusive endophytism proof would be needed from re-isolation of the bacterium in the root and a molecular comparison with the inoculum used.

4. Discussion

Inoculants based on PGPR are becoming increasingly important as potential alternatives to chemical fertilization, since they are considered as ecologically acceptable and economically attractive. These inoculants interact positively with plants and may perform vital functions by various mechanisms, such as carbon transformations, nutrient recycling, regulation of biological populations and maintenance of soil structure [48–50]. Among these bacteria, *B. subtilis* subsp. *spizizenii* has been shown to increase phosphate solubilization, to synthesize growth regulators and to act as a bio-controller of common soil-borne phytopathogens such as the *Fusarium* and *Phytium* fungi as well [20].

Most bio-inoculants grow as bacterial vegetative forms, i.e., as free-living planktonic bacteria; however, some of them, including *B. subtilis* subsp. *spizizenii*, have been shown to be able to grow both in the planktonic form or as a biofilm [27,51]. Species that grow predominantly as biofilms, such as *B. subtilis*, are thought to possess advantages in terms

of reproducing and surviving in the environment [52]. Within the biofilm, different cell subpopulations coexist that show different functions, e.g., matrix-producing cells, vegetative cells, cannibal cells or spore-forming cells. Biofilm vegetative cells are protected and, once detached, are able to colonize new niches, similar to how the planktonic cells act. Moreover, spores are resistant and survive in more adverse conditions compared to vegetative cells. Thus, again, spore formation gives *B. subtilis* competitive advantages over other soil bacteria [26,53].

Several works have analyzed populations of vegetative cells and spores in biofilm and planktonic forms [20,54–56]; the results were consistent with the present study, which showed that spores were in a higher proportion in the biofilm compared to the planktonic inoculum. Moreover, it has been observed that biofilms of *B. subtilis* can develop external projections or fruiting bodies, which can enhance spore dispersion [54,55]; in the sequence, the spores could colonize new regions after turning into vegetative cells [53]. Therefore, a higher spore load of a biofilm ensures its greater persistence and resilience against fluctuations in the environment compared with a planktonic inoculum.

Biofilm stability is important to protect it against degradation. Greater biofilm stability provides an important advantage as it increases the shelf life of the inoculant. Moreover, biofilm degradation has been shown to depend on the matrix structure [57] and also on processes involving the action of different enzymes [58]. Our results showed that the carbon source had an effect on the biofilms, so that the cultures of *B. subtilis* subsp. *spizizenii* growing on a glycerol medium had increased stability compared to those growing on a glucose medium. Similar results have been observed in previous works conducted under laboratory conditions, even leading to dispersion when mild starvation occurred [53].

Another important aspect is that the positive effects of a bio-inoculant also depend on the interaction between the bacterial strain and the target crop. This is because each plant variety produces molecules that stand up for colonization and the multiplication of specific microorganism strains [59,60]. For example, seed inoculation of four strains of *Bacillus* showed different response in tomato and pepper [36], while seed inoculation of the tomato variety Florardade with three strains of *Pseudomonas fluorescens* resulted in beneficial, null or negative effects [37]. Moreover, inoculation with *Enterobacter aerogenes* increased the germination power of sunflower seeds by 30% with respect to a control, but it had no effect on papaya [61]. Therefore, a given PGPR may produce an advantageous effect on one type of plant but not on another one, and the effects may even be different between varieties of the same species.

The International Seed Testing Association [62] describes the seed germination process as the initiation of a metabolically active state, which is physiologically revealed by cell division and differentiation. Liquid inoculation with the planktonic form requires an adequate contact time between the seed and bio-inoculant to guarantee a positive plant development response [38,42]. Imbibition times may vary from 20 min to several hours according to the seed and type of bacteria studied [63–66]. Several surveys have tested contact times of tomato inoculated with *Bacillus* sp., [67], and the results show that 30 min is the most suitable imbibition time; therefore, this finding was used in the present work. To increase the low chance of survival of planktonic cells in liquid formulations, encapsulation in various carriers (alginates, chitosans, etc.) has been proposed, which protects them from environmental stress situations; in addition, the use of adhesive materials has been recommended to facilitate contact of planktonic inoculants with seeds [68]. However, after biofilm inoculation, adherence between bacteria and seeds is often observed, which can allow greater interaction. This adherence could be due to the action of the biofilm's exopolysaccharides together with various products that originate from the bacteria, such as hormones, and from the seed wall, such as mucilage [61].

Based on the previous framework, the effects of inoculation on the different growth stages of tomato are next addressed. In our work, the germination rate was significantly higher for the two types of inoculation than for the control; moreover, the inoculum effects on germination were independent of the variety of *S. lycopersicum*. This is consistent with

a previous study, which also tested the effects of *Bacillus* on tomato germination [36]. In addition, the biofilm inoculation resulted in a retarding of germination after 5 days but not after 10 days. This initial delay could be explained by the biofilm initially acting as a barrier between the seed and the environmental signals that start germination, namely humidity, temperature and gaseous phase composition [69]. Once this barrier is overcome, the biofilm exopolysaccharide matrix allowed closer contact with the bacteria. With respect to radicle and hypocotyl development, for the Platense variety, the biofilm had a positive effect on the radicle but not on the hypocotyl length; moreover, the hypocotyl length showed no significant differences between the inoculated and non-inoculated treatments. However, for the Río Grande variety, there were positive effects of the biofilm inoculation on the radicle and hypocotyl lengths. The different responses to the inoculum treatment between varieties could be linked to the non-identical effects of auxin, indole acetic acid (IAA) and cytokinin zeatin riboside acting as growth regulators [20]. More developed tomato seedlings are expected to be more unaltered by potential transplanting stress [70], which results in advantages in terms of increasing growth and production.

At the end of the vegetative growth stage, again, the effects on shoot and root biomass showed a dependence on plant variety [36]. The plant-growth-promoting effect of the two inoculant methods tested was superior for the Río Grande than for the Platense variety. Also, the biofilm inoculation performed better than planktonic inoculation at this growth stage. Again, these results could be associated with increases in growth regulator (auxin, IAA, etc.) production, phosphate solubilization and factors related in general to increased nutrient uptake following the inoculum application [20].

At harvest time, the inoculation positively affected several growth variables (height, dry shoot and dry root biomass, leaf area) and fruit production of the Río Grande tomato plants. Regarding the inoculation methods, the application of *B. subtilis* subsp. *spizizenii* as a biofilm provided the best results for the vegetative and productive variables, so that shoot and root biomass and fruit production showed significant increases with respect to the planktonic inoculum. The quantity and characteristics of fruits are the most important aspects in tomato production and quality [71]. Our work showed that the inoculation with biofilm was not only beneficial for plant growth but also for plant productivity and fruit quality. Other variables such as plant height, fruit weight and soluble solids were also greater in the biofilm than in the planktonic treatment, although the differences were not significant. Eventually, the leaf chlorophyll content was similar between the control and inoculated treatments; this was an expected result, as chlorophyll is strongly related to plant nitrogen content [72], and *B. subtilis* subsp. *spizizenii* has not been demonstrated to act as a nitrogen-fixing bacterium.

Retrieving the *B. subtilis* subsp. *spizizenii* at harvest time from inside the roots of the *S. lycopersicum* variety Río Grande after seed inoculation with the biofilm indicated that the bacterium behaved as an endophytic one. Therefore, starting from the biofilm, the bacterium was first able to colonize the seed, and then it migrated and infected the seedling roots in a significant number, granting access, proliferation and persistence in the plant along the whole growth cycle. These results underpin the advantages of *B. subtilis* biofilm as a bio-inoculant, as they reveal the ability of the inoculated bacterium to infect and remain viable in the crop until harvest time. It is noteworthy that not all PGRPs have been found to persist after initial infection and colonization, because the conditions within plants can be suppressive to bacterial growth [73]. Thus, endophytic bacteria show particular promise as bio-inoculants, since they may act in a specialized niche within plants, functioning as biocontrol agents [20,74,75]. However, conclusive proof of endophytism based on the molecular comparison of bacteria re-isolated from the root at harvest time with the inoculum was beyond the scope of this work. Again, the main innovation that has arisen from our work lies in the way in which the biofilm was inoculated on the tomato seeds, which differed from that used until now. For example, earlier biofilms from *Bacillus* sp., *Pseudomonas* sp. or a mixture of both strains have been inoculated on tomato [76], and a biofilm from *Streptomyces* supplied with perlite has also been applied to *Triticum*

aestivum [77]; in either case, the bacteria was not able to fulfill their positive effects from the very beginning of seed germination.

In summary, the present work highlights the advantages of biofilm incorporation as a seed inoculant for increasing tomato crop productivity and quality. Moreover, biofilm application on seeds can be explored as an effective substitute for inorganic fertilizers and pesticides due to its proven effects in promoting nutrient availability and plant growth and in protecting plants from biotic and abiotic stresses. Therefore, seed inoculation using biofilm also involves environmental sustainability with regards to agriculture and soil conservation, as this practice can preserve soil chemical and physical integrity and provide maintenance of crop yields in the future.

5. Conclusions

To compare the effects of tomato seed inoculation with either conventional planktonic or with biofilm cultures of *B. subtilis* subsp. *spizizenii* on vegetative and productive variables of tomato, two independent experiments were carried out under laboratory and greenhouse conditions; the former experiment involved two tomato varieties, and the latter involved one variety. The experimental design consisted of a non-inoculated treatment and two different inoculation treatments.

Under laboratory conditions, two different tomato varieties, namely Platense and Río Grande, were studied after germination and at the end of the vegetative growth period. Different responses to the inoculum treatment between the varieties were obtained, showing that the Río Grande variety performed better than the Platense variety. Specifically, the biofilm seed inoculation of the Río Grande variety resulted in a significantly greater radicle and hypocotyl length and a higher root and aerial biomass compared to the planktonic seed inoculation.

Under greenhouse conditions, the seed inoculation of the Río Grande variety showed that at harvest time, the biofilm significantly increased the root and shoot biomass and fruit number compared to the planktonic treatment. The plant height, fruit weight and soluble solids were also greater in the biofilm treatment than in the planktonic treatment, although the differences were not significant.

Overall, the proposed inoculation method, based on a biofilm of *B. subtilis* subsp. *Spizizenii*, allowed the bacteria to perform their positive effects at different growth stages, from seed germination to harvest time, and it was superior to the traditional planktonic inoculation method.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments11030054/s1, Figure S1: Biofilm from *B. subtilis* subsp. *spizizenii* at the air–liquid interface. (Cultivation in minimum salt medium (MSM) plus 55 mM glutamic acid and 1% glycerol under static conditions at 30 °C for 96 h); Figure S2: *S. lycopersicum* plants, Río Grande variety, at harvest time (4 months). (a) Planktonic inoculum treatment, (b) non-inoculated treatment; Figure S3: *S. lycopersicum* plants, Río Grande variety, at harvest time (4 months). (a) Biofilm treatment, (b) non-inoculated treatment; Figure S4. *S. lycopersicum* plants, Río Grande variety, at harvest time (4 months). (a) Planktonic inoculum treatment, (b) Biofilm treatment.

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